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14. ABSTRACT

We have focused upon tissue analyses and novel agents for prostate cancer. We have collected close to one thousand cases of prostate cancer tissue samples. DNA samples were extracted from 250 cases. Genotyping of various SNPs revealed a SNP from EGFR and another SNP from MMP7 were significantly associated with prostate cancer recurrence, by uni or multi-variate analyses. Ongoing experiments led by these findings aim to determine inhibitors of EGFR (Tarceva) or MMP7 (Marimastat) enhance therapeutic efficacy of radiotherapy and chemotherapy in prostate cancer models. We also investigate small molecule compounds such as aurora kinase inhibitors in radiosensitizing prostate cancer models.

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1. Introduction:

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2. Body:

Establishing clinical database:

We have established a database for close to one thousand prostate cancer patients who underwent prostatectomy at Vanderbilt University Medical Center since 1997. The study was approved by Institutional Review Board (IRB# 030986) at Vanderbilt University School of Medicine. All patients had clinically localized prostate cancer and were treated with radical prostatectomy as a primary treatment. All patients had adenocarcinoma confirmed histologically. The patients were followed at Vanderbilt Hospital or at local hospitals. All pathological information was reviewed by one pathologist and the tumor differentiation was evaluated using Gleason`s score criteria. Clinical stage was classified according to the AJCC TNM staging system. The Clinical and histological characteristics of the patients are tabulated.

Tissue preparation and DNA extraction

Using a standard microtome with disposable blades, 5um thickness sections of a representative areas of normal prostatic glands were cut from the paraffin embedded blocks and stained with Hematoxylin and eosin (H&E) and then examined under a microscope to verify the absence of prostate cancer. 5um thickness section (about 1ug) from each patient was used for DNA extraction. The sections were deparaffinized with xylene at room temperature for 30minutes twice. Then the deparaffinized tissue was washed with 100% ethanol twice. After the ethanol had evaporated completely, the tissue was completely lysed with proteinase K. Then QIAamp DNA Mini Kit (QIAGEN Inc, Valencia, CA) was used to extract and purify 250 DNA samples from the tissues according to the tissue protocol of the kit.

Polymorphism Genotyping

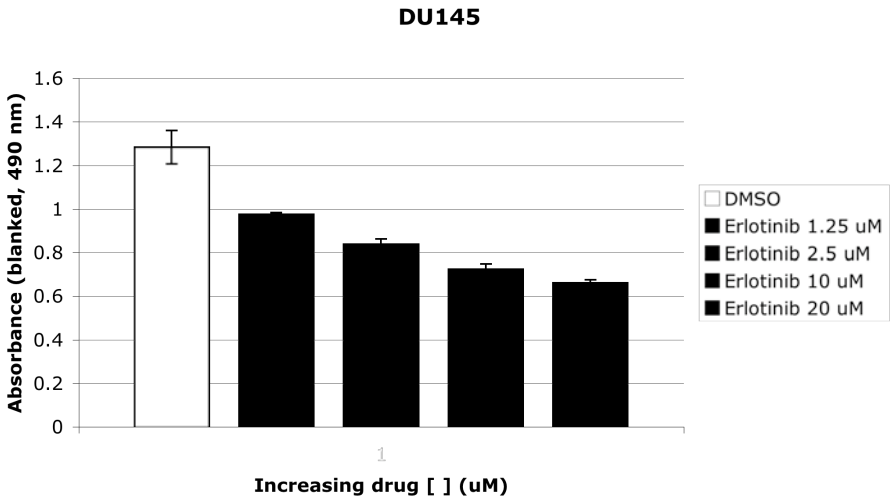
The allelic discrimination of various gene polymorphisms was assessed with the ABI Prism 7900 HT Sequence Detection System. Polymerase chain reaction was performed
with a total volume of 5 _L, which contained approximately 2.5 ng DNA, 1 _ Taqman Universal PCR Master Mix, each primer at a concentration of 900 nM, and each probe at a concentration of 200 nM. The fluorescence levels were measured with an ABI PRISM 7900 HT Sequence Detector and resulted in clear identification of three genotypes of each polymorphism. The laboratory staff was unaware of the identity of the men. Quality controls were included in the genotyping assays. Each 384 well plate contained four water, eight CEPH 1347-02 DNA, and eight blinded quality control samples, and unblended quality control samples. The blinded and unblended quality control samples were taken from the second tube of the study samples included in this study.

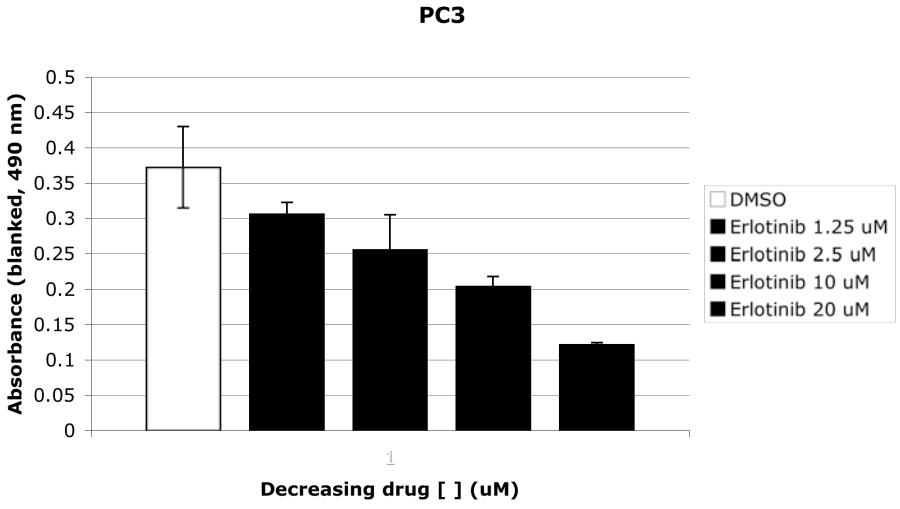
EGFR:

Genomic DNA was extracted from the resected prostate tissues, and genotyped with allele-specific Taqman probes for nine haplotype-tagging single nucleotide polymorphisms (htSNPs) in intronic, exonic, and flanking regions of linkage disequilibrium in the EGFR gene. Correlations between alleles and clinical markers, as well as follow-up recurrence and survival data up to ten years post-prostatectomy were investigated using univariate and multivariate genetic analysis models. There was a statistically-significant association between the htSNP rs884419, located 1,249 base pairs downstream

of the EGFR locus, and prostate cancer recurrence, defined in the study by at least a PSA biochemical recurrence ($P < 0.001$, log-rank test). At three years post-prostatectomy, 80% of patients with the unfavorable genotype (EGFR rs884419 A/A) had developed recurrent prostate cancer; only 20% of patients with the favorable genotypes (EGFR rs884419 G/G or EGFR rs884419 A/G) had a detectable recurrence at the time. The frequency of the unfavorable allele in the study population was 3%. Based on Cox proportional hazards analysis, the favorable genotypes G/G and A/G confer a hazard ratio for developing prostate cancer recurrence of 0.09 (0.02 to 0.4, 95% CI) and 0.12 (0.03 to 0.44, 95% CI), respectively ($P = 0.001$). These data suggest that a polymorphism flanking the EGFR gene is an independent prognostic marker to predict biochemical recurrence after radical prostatectomy. For prostate cancer patients with the unfavorable EGFR rs884419 biomarker, optimizing the effects of radiation therapy may positively impact prognosis. Our findings support EGFR as a molecular target in prostate cancer, and ongoing preclinical studies are evaluating EGFR signaling inhibition as a strategy to enhance radiation sensitivity of prostate cancer cells.

Erlotinib (Tyrosine kinase inhibitor of EGFR) - mediated EGFR inhibition in prostate cancer causes a dose-dependent reduction of cellular proliferation: As shown below, DU145 or PC3 prostate cancer cell lines were seeded in 96-well plates (2,000 cells/well) and treated with various doses of Erlotinib (1.25 μM-20μM) or control (DMSO) for 74 h. Cell proliferation was studied using the non-radioactive cellular proliferation assay CellTiter 96®. Bars represent the mean absorbance values for each treatment condition and error bars indicate the standard deviation (n=3; *, P<0.0005, student's t-test). Therefore, EGFR is a target for prostate cancer therapy.





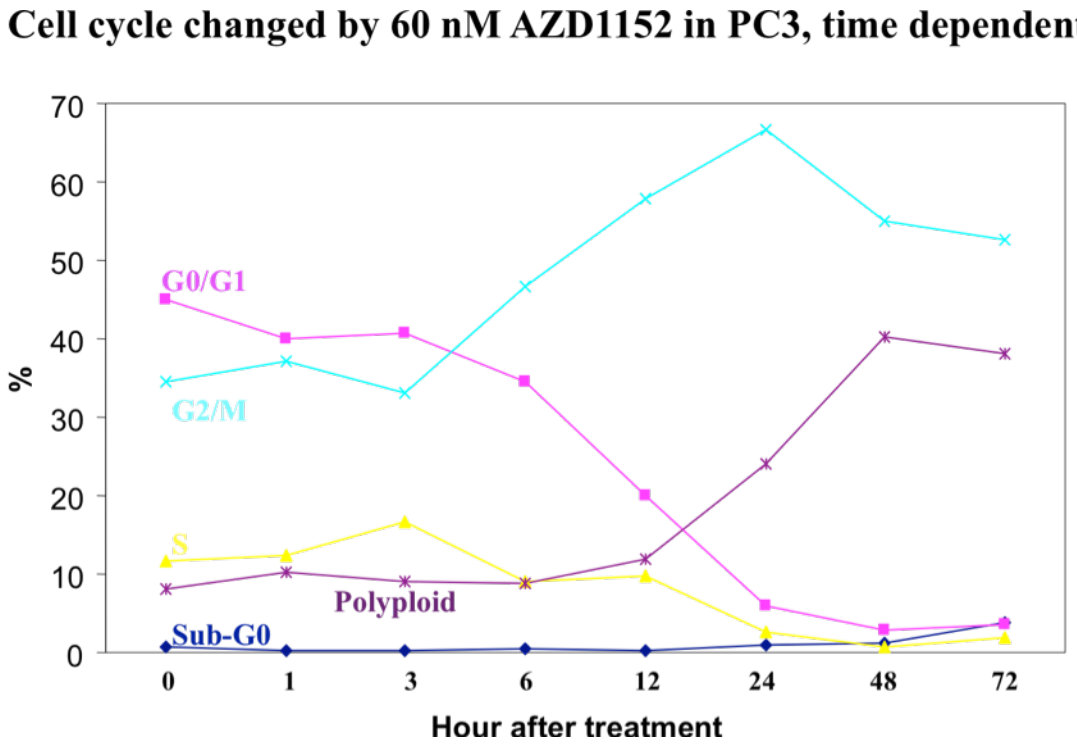
MMP7:

The single nucleotide polymorphisms within various regions of the MMP7 gene were assessed with correlation to age at diagnosis, margin status, extracapsular extension, lymph node metastasis, local recurrence and tumor survival in paraffin-embedded prostate tissue specimens from patients with early-stage prostate cancer receiving radical prostatectomy alone. The rs10895304 polymorphism was the sole significant polymorphism. The SNP correlated to increased recurrence rates in post-prostatectomy patients ($p<0.0001$, Log Rank Test). The frequency of the homozygous dominant (AA) is 74%, the heterozygote (A/G) is 20% and the homozygous recessive (G/G) is 6%. Multivariate analysis (using Chi square) did not detect a confounding relationship between recurrence and age at diagnosis, PSA or Gleason's score. None of the other assayed polymorphisms were significant, and no correlations were made to other clinical variables. The rs10895304 polymorphism is predicted of increased local recurrence risk in patients with clinically localized prostate cancer. For this subset of patients, prostatectomy alone may not be adequate for local control. This is a novel and relevant marker that should be evaluated for improved risk stratification of patients who may be candidates for early post-operative radiation therapy to improve local control.

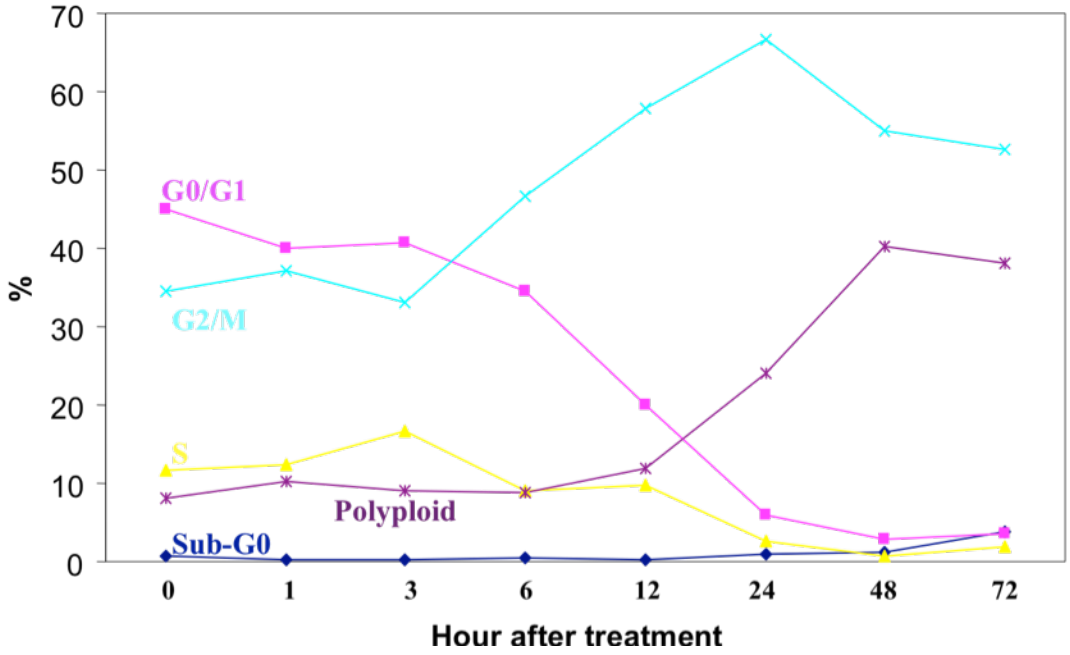
Ongoing experiments will test the therapeutic efficacy of MMP7 inhibitors in prostate cancer models.

Aurora kinase inhibitors as biomarkers and targets for prostate cancer therapy:

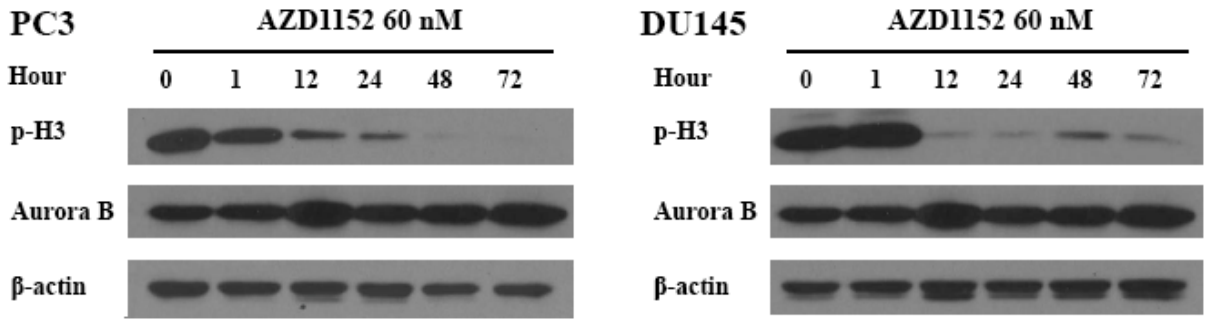
We are using similar approaches in genotyping SNPs in the genes for aurora kinase A and B. We also tested an inhibitor of aurora kinase B, AZD1152, in prostate cancer models. We found that AZD1152 induces G2/M arrest and polyploidy cells, as shown below per flow cytometry analyses:



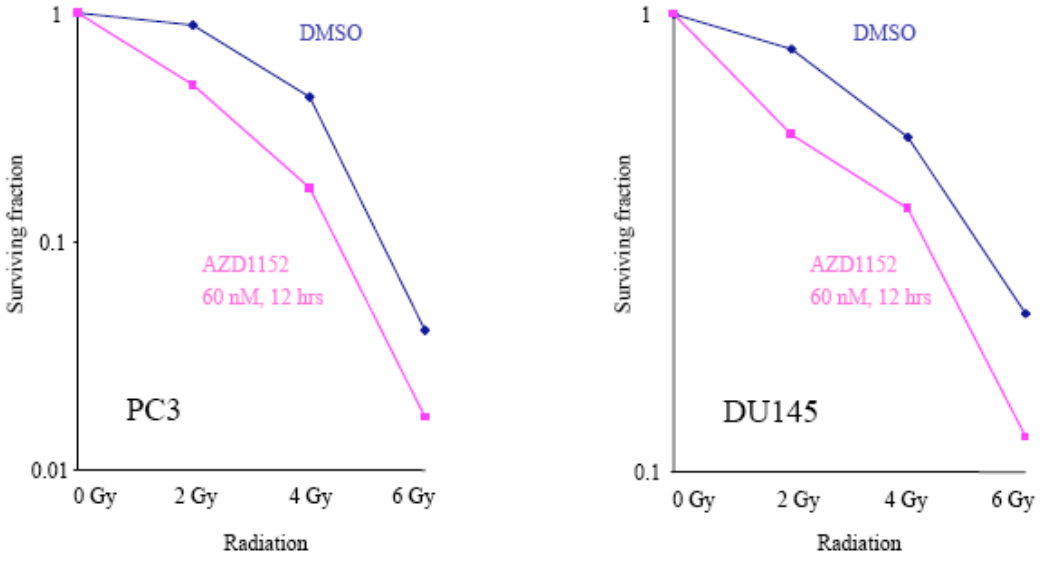
Cell cycle changed by 60 nM AZD1152 in PC3, time dependent



We then used western analyses of the substrate of aurora kinase B, phospho-histone 3, being inhibited by AZD1152, as shown below:



Finally, clonogenic study demonstrated that inhibition of aurora kinase B by AZD1152 sensitized prostate cancer cells to radiation, as shown below:



3. Key Research Accomplishments:

1). SNPs of EGFR and MMP7 as biomarkers for prostate cancer recurrence following prostatectomy.
2). EGFR and MMP7 as potential targets for prostate cancer therapy.
3). Aurora kinase inhibitors as radiosensitizers by cycle-cycle arrests.

4. Reportable outcomes:

1. Cao C, Subhawong T, Albert JM, Kim KW, Geng L, Sekhar KR, Gi YJ, **Lu B**. Inhibition of mammalian target of rapamycin or apoptotic pathway induces autophagy and radiosensitizes PTEN null prostate cancer cells. *Cancer Res. 2006 Oct 15;66(20):10040-7.*

2. Kwang Woon Kim, Robert W. Mutter, Carolyn Cao, Jeffrey M. Albert, Michael Freeman, Dennis E. Hallahan, and **Bo Lu.** Autophagy for cancer therapy through inhibition of proapoptotic proteins and mTOR signaling. *J Biol Chem. 2006 Dec 1;281(48):36883-90.*

3. Eric Shinohara, Kenneth Niermann, Carolyn Cao, Fenghua Zeng, Dennis E. Hallahan, and Bo Lu. mTOR inhibitors as a potential anti-angiogenesis agent enhanced efficacy of radiotherapy. *Oncogene 2005 24: 5414-5422.*

4. Robert Lee Browning, Hecheng Li, Eric T Shinohara, Qiuyin Cai, Heidi Chen, Regina Courtney, Carolyn Cao, Wei Zheng, and **Bo Lu** ATM polymorphism IVS62+60G>A is not associated with aggressiveness of disease in prostate cancer. *Urology. 2006 Jun;67(6):1320-3.*

5. Li HC, Eric Shinohara and Bo Lu. Endostatin polymorphism 4349G/A(D104N) is not Associated with Aggressiveness of Disease in Prostate Cancer *Dis Markers* Volume 21, Number 1, 2005

6. Li HC, Eric T Shinohara and Bo Lu Plasminogen activator inhibitor-1(PAI-1) promoter polymorphism is not associated with prognosis in prostate cancer. *Clinical Oncology, Volume 18, Issue 4, May 2006, Pages 333-337.*

5. Recent Publications:

1). Luigi Moretti, Kwang Woon Kim, and **Bo Lu**. Crosstalk between Bak/Bax and mTOR signaling regulates radiation-induced Autophagy. Autophagy 2007 Apr-Jun;3(2):142-4.

2). Luigi Moretti, Yong Cha, Kenneth Niermann, and **Bo Lu**. Switch between Apoptosis and Autophagy: Radiation induced-Endoplasmic Reticulum Stress? Cell Cycle 6(7), 2007 April 1.

3). Jeffrey Albert, Carolyn Cao, Alan Sandler, David Johnson, Jennifer Low, Mace Rothenberg and **Bo Lu**. Inhibition of poly(ADP-ribose) polymerase enhances cell death and improves tumor growth delay in irradiated lung cancer models. (in print, Clinical Cancer Research).

4). Kwang Woon Kim, Robert W. Mutter, and **Bo Lu.** Inhibition of survivin and Aurora B kinase sensitizes mesothelioma cells by enhancing mitotic arrests. (in print, Int J Radiat Oncol Biol Phys).

5). Cao C, Subhawong T, Albert JM, Kim KW, Geng L, Sekhar KR, Gi YJ, **Lu B**. Inhibition of mammalian target of rapamycin or apoptotic pathway induces autophagy and radiosensitizes PTEN null prostate cancer cells. Cancer Res. 2006 Oct 15;66(20):10040-7.

6). Kwang Woon Kim, Robert W. Mutter, Carolyn Cao, Jeffrey M. Albert, Michael Freeman, Dennis E. Hallahan, and **Bo Lu.** Autophagy for cancer therapy through inhibition of proapoptotic proteins and mTOR signaling J Biol Chem. 2006 Dec 1;281(48):36883-90.

7). Kwang Woon Kim and **Bo Lu**. Stat3 mediates transcriptional downregulation of survivin following irradiation. Molecular Cancer Therapeutics 5(11): 2659-2665, 2006.

8). Carolyn Cao, Jeffrey Albert, Alan Sandler, David Johnson, and **Bo Lu**. Vascular Endothelial Growth Factor Tyrosine Kinase Inhibitor AZD2171 and Fractionated Radiotherapy in Mouse Models of Lung Cancer. Cancer Res 2006 66: 11409-11415.

9). Jeffrey M. Albert, Carolyn Cao, Ling Geng, Lauren Leavitt, Dennis E. Hallahan and **Bo Lu**. Integrin $\alpha_v\beta_3$ antagonist Cilengitide enhances efficacy of radiotherapy in endothelial cell and non-small cell lung cancer models. Int J Radiat Oncol Biol Phys 2006, 65:1536-1543.

10). Jeffrey M. Albert, Kwang Woon Kim, Carolyn Cao, and **Bo Lu.** Targeting the Akt/mammalian target of rapamycin pathway for radiosensitization of breast cancer. Mol Cancer Ther 2006 5: 1183-1189.

11). Carolyn Cao, Eric T. Shinohara, Ty K. Subhawong, Ling Geng, Kwang Woon Kim, Jeffrey M. Albert, Dennis E. Hallahan, and **Bo Lu** Radiosensitization of lung cancer by nutlin, an inhibitor of murine double minute 2 Mol Cancer Ther 2006 5: 411-417

12). Carolyn Cao, Kenneth Niermann and **Bo Lu**. Radiation sensitization of lung cancer and its angiogenesis through inhibition of Clusterin. Int J Radiat Oncol Biol Phys 2005 63:1228-1236.

13). Robert Lee Browning, Hecheng Li, Eric T Shinohara, Qiuyin Cai, Heidi Chen, Regina Courtney, Carolyn Cao, Wei Zheng, and **Bo Lu** ATM polymorphism IVS62+60G>A is not associated with aggressiveness of disease in prostate cancer Urology. 2006 Jun;67(6):1320-3.

14**).** H. Li, E.T. Shinohara, Q. Cai, H. Chen, R. Courtney, C. Cao, Z. Wang, M. Teng, W. Zheng and **B. Lu.** Plasminogen Activator Inhibitor-1 Promoter Polymorphism is Not Associated With the Aggressiveness of Disease in Prostate Cancer. Clinical Oncology, Volume 18, Issue 4, May 2006, Pages 333-337.

15). Li HC, Albert JM, Shinohara ET, Cai Q, Freyer A, Cai H, Cao C, Wang Z, Kataoka N, Teng M, Zheng W, **Lu B**. E-cadherin promoter polymorphisms are not associated with the aggressiveness of prostate cancer in Caucasian patients. Urol Oncol. 2006 Nov-Dec;24(6):496-502.

16). Carolyn Cao, Eric Shinohara, Kenneth Niermann, Dennis Hallahan, and **Bo Lu**. MDM2 as a radiosensitizing target for lung cancer and its vasculature. Mol Cancer Ther 2005 4: 1137-1145.

17). Eric Shinohara , Kenneth Niermann, Carolyn Cao, Fenghua Zeng, Dennis E. Hallahan, and **Bo Lu**. mTOR inhibitors as a potential anti-angiogenesis agent enhanced efficacy of radiotherapy. Oncogene 2005 24: 5414-5422